

Elemol and amyris oil repel the ticks *Ixodes scapularis* and *Amblyomma americanum* (Acari: Ixodidae) in laboratory bioassays

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Abstract The essential oil from *Amyris balsamifera* (Rutaceae) and elemol, a principal constituent of the essential oil of Osage orange, *Maclura pomifera* (Moraceae) were evaluated in in vitro and in vivo laboratory bioassays for repellent activity against host-seeking nymphs of the blacklegged tick, *Ixodes scapularis*, and the lone star tick, *Amblyomma americanum*. Both bioassays took advantage of the tendency of these host-seeking ticks to climb slender vertical surfaces. In one bioassay, the central portion of a vertical strip of filter paper was treated with test solution and ticks placed or allowed to crawl onto the untreated lower portion. In the other bioassay, a strip of organdy cloth treated with test solution was doubly wrapped (treatment on outer layer) around the middle phalanx of a forefinger and ticks released on the fingertip. Both amyris oil and elemol were repellent to both species of ticks. Elemol did not differ significantly in effectiveness against *A. americanum* from the widely used repellent deet. At 2 and 4 h after application to filter paper, 827 µg amyris oil/cm² paper repelled 80 and 55%, respectively, of *A. americanum* nymphs. *Ixodes scapularis* was repelled by lower concentrations of amyris oil and elemol than *A. americanum*.

Keywords *Amyris balsamifera* · *Maclura pomifera* · Blacklegged tick · Lone star tick

Introduction

Tick-borne diseases pose a serious threat to humans in most parts of the habitable world (Sonenshine 1993; Parola and Raoult 2001). In the United States, the blacklegged tick,

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Ixodes scapularis Say, and lone star tick, *Amblyomma americanum* (L.) are responsible for many tick bites of humans and resultant disease transmission. The former species is the principal vector of *Borrelia burgdorferi*, the pathogen causing Lyme disease in eastern and central United States (Spielman et al. 1985), and the latter transmits *Ehrlichia chaffeensis*, the causative agent of human monocytic ehrlichiosis (Childs and Paddock 2003). Larvae, nymphs and adults of these species bite humans. However, it is the nymphal stage of *I. scapularis* that is responsible for most Lyme disease infections in humans. Where *A. americanum* occurs, its aggressive host-seeking behavior tends to make it highly visible to the public (Armstrong et al. 2001).

Recently developed area-wide tick control technologies have shown promise, but their implementation has lagged (Fish and Childs 2009; Dolan et al. 2004; Piesman and Eisen 2008). Consequently the use of repellents is recommended for the personal protection of people entering tick habitats (CDC 2002). Permethrin-based products applied to clothing have proven effective in repelling *A. americanum* and *I. scapularis* (Schreck et al. 1982; Lane and Anderson 1984). Since the 1950s, products containing deet (*N,N*-diethyl-3-methyl benzamide) have been the mainstay for use on human skin. Some deet formulations provide lasting protection from ticks (Carroll et al. 2008). With the discovery of effective synthetic alternatives to deet-based products (e.g., picaridin, IR3535) in recent years, there has been a growing interest in discovery of tick repellents from natural sources (e.g., Jaenson et al. 2005; Tunón et al. 2006; Garboui et al. 2007; Carroll et al. 2007; Bissinger et al. 2009).

Evidence of the repellent activity contained in sesquiterpene-rich essential oils and their purified isolates and/or compounds (from heartwood, bark, leaves, etc.) has appeared in the literature over the last 10 years, with a recent focus on sesquiterpenes containing alcohol, aldehyde, ketone, and acid moieties from extractions of white cypress pine, *Callitris glauophylla* Thompson et Johnson, Japanese cedar, *Cryptomeria japonica* (L. f.) D. Don, the American beautyberry bush, *Callicarpa americana* L., Alaska yellow cedar, *Chamaecyparis nootkatensis* D. Don, a Malaysian member of the custard apple family, *Goniothalamus uvatioides* King, Osage orange, *Maclura pomifera* (Raf.) Schneid, amyris, *Amyris balsamifera* L., and Siam-wood, *Fokienia hodginsii* (Dunn) Henry and Thomas (Ahmad and Jantan 2003; Watanabe et al. 2005; Wang et al. 2006; Carroll et al. 2007; Dietrich et al. 2006; Schultz et al. 2006; Paluch et al. 2009). Availability of these essential oils and extracts can be limited, but some are supplied by commercial sources.

Sesquiterpenes occurring in balsam torchwood, *A. balsamifera* (Rutaceae) and elemol, a major constituent of the essential oil of Osage orange (Moraceae), have been shown by Paluch et al. (2009) to repel the yellow fever mosquito, *Aedes aegypti* (L.). The purpose of this study was to ascertain whether the repellent properties of *Amyris* essential oil and elemol extended to ixodid ticks. To this end, we tested host-seeking *A. americanum* and *I. scapularis* against amyris oil and elemol in *in vitro* and *in vivo* laboratory bioassays.

Materials and methods

Ticks

Larvae of *I. scapularis* obtained from a colony at Oklahoma State University were fed on rats (in accordance with USDA, ARS, Beltsville Area Animal Care and Use Committee Protocols #05-022 and #08-013). The fed larvae were held in vials at 23–24°C, ≈ 97% RH and a photoperiod of 16:8 h (L:D). Nymphs of *A. americanum* were from a colony

maintained at the USDA, ARS, Knippling-Bushland U. S. Livestock Insects Research Laboratory, Kerrville, TX and held at 23–24°C, ≈ 97% RH and a photoperiod of 16:8 h (L:D). The *I. scapularis* and *A. americanum* nymphs were used in bioassays 2–4 months after eclosion.

Chemicals

Amyris (*A. balsamifera*) essential oil was purchased from commercial sources (Sigma-Aldrich, St. Louis, MO; Phoenix Natural Products, Middlesex, England). Sufficient quantities of purified elemol were isolated from technical grade materials in the laboratory at Iowa State University. A supply of technical grade, 55% purity elemol (Augustus Essential Oils, Hampshire, England) was further purified via column chromatography with silica gel, 40–140 mesh (J.T. Baker, Phillipsburg, NJ), to ≥ 80% purity using hexane/diethyl ether (9:1) mobile phase. Further column purification to ≥ 95% was achieved using a hexane/acetone/diethyl ether (8:1:1) mobile phase system.

Purity of samples was assessed on a Hewlett Packard 5890 Series II gas chromatograph with a 30 × 0.25-mm i.d. × 0.25 µm film thickness DB-WAX column (Alltech, Deerfield, IL) with flame ionization detection. The injector temperature was 250°C, and the split valve was opened 1 min after injection. The oven initial temperature was set at 120°C for 1 min, and then increased at 4°C/min to 236°C. Confirmation of compound identity was completed on a Hewlett Packard 5972 Mass Selective Detector (MSD). Mass spectra were recorded from 30 to 550 a.m.u. with electron impact ionization at 70 eV. Chemical identification was assigned to elemol and amyris essential oil compounds detected, and results confirmed by comparison of the retention indices with reference spectra in a mass spectral library (Wiley 138 K, John Wiley and Sons) and comparison to literature sources (Van Beek et al. 1989). Deet was purchased from Sigma-Aldrich.

Bioassays

An in vitro and an in vivo bioassay were used to evaluate the repellency of elemol and amyris oil. The in vitro bioassay (vertical filter paper bioassay) took advantage of the tendency of host-seeking ticks to climb slender vertical surfaces (Carroll et al. 2004). A 4 × 7-cm rectangle of Whatman No. 4 filter paper was marked with a pencil into two 1 × 4-cm zones at either of the far ends of the paper with an intervening 4 × 5-cm zone. Test solutions (165 µl) were evenly applied by pipettor to the 20-cm² central zone of the filter paper strip. The strip was allowed to dry for 10–15 min and suspended from a bulldog clip hung from a slender dowel held by an Aptex No. 10 double clip work holder (Aptex, Bethel, CT). The vertical strip hung over a Petri dish (9 cm diameter) that had been glued in the center of a 15-cm diameter Petri dish with water creating a moat between their walls (1.5 cm high). A storage vial containing ticks was opened in the center of moated Petri dishes (5.5 and 9 cm diameters). When *A. americanum* nymphs had crawled onto the rim of the vial and the Petri dish, the strip was removed from the peg and held so that a total of 10 ticks crawled onto the lower untreated zone. With *I. scapularis*, the nymphs were transferred to the filter paper using forceps.

Locations of the ticks were recorded at 1, 3, 5, 10, and 15 min after the tenth tick was on the filter paper. We scored repellency based on the locations of ticks at 15 min. Ticks were considered repelled if they remained on the lower untreated zone of the filter or if they dropped off the strip without having crossed into the upper untreated zone. The moated

Petri dish beneath the strip confined ticks that dropped from the paper. Ticks that climbed to the upper untreated zone were removed to prevent their return to the lower zones.

The in vivo bioassays (fingertip bioassay with double-wrapped cloth) were conducted in compliance with a human-use protocol (#2007-240) reviewed and approved by the MedStar Research Institute Institutional Review Board. This modified fingertip bioassay, was described in detail and depicted by Carroll et al. (2005). A strip of organdy (7×7 mesh/mm) (Hancock Fabrics, Laurel, MD) was cut in the shape of a hockey stick (9 cm long section, 4.5 cm short section, 4–4.5 cm wide) so that it could be wrapped twice around the index finger of JFC with only the outer layer receiving test solution. The boundary of an area of the cloth corresponding to the area between the first and second joints of the finger was marked with a lead pencil and served as the treatment area. The volume of the solution applied to the cloth was based on the dimensions of the left index finger. The volume required for the desired nmoles/cm² cloth was calculated from the average of the circumferences of the two finger joints multiplied by distance between the deepest crease of each joint.

While an organdy strip was partly supported by the rim of a glass petridish, 52 µl of test solution was evenly distributed on the treatment area with a pipettor. After allowing 10–15 min for the cloth to dry, it was doubly wrapped around the index finger, so that the treated portion of the cloth completely encircled the finger and covered the entire second phalanx. An untreated portion of the cloth extended 5–6 cm beyond the first joint toward the base of the finger. To hold the cloth in place three small dabs of beeswax were smeared on the upper surface of the inner layer of cloth where layers overlapped and pressure from another finger applied to adhere the layers. Because *I. scapularis* nymphs tended to be slower, were more apt to drop from untreated skin, and had a far larger percent remain immobile than *A. americanum* nymphs, it was necessary to screen the former for tenacity and readiness to climb (Schreck et al. 1995). While the test solution dried on the cloth, *I. scapularis* nymphs were placed on the tip of the untreated index finger held vertically. Those ticks that climbed ≈ 0.5 were used in the bioassay. Using forceps, 10 nymphs of *I. scapularis* were placed on the untreated fingertip near the base of the nail. As in the vertical filter paper bioassay, 10 *A. americanum* nymphs were allowed to crawl from the rim of an open vial and moated Petri dish onto the fingertip. Once 10 ticks were on the fingertip, the finger was tilted slowly until vertical with the tip downward. The locations of the ticks were recorded at 1, 3, 5, 10, and 15 min after the tenth tick was on the finger. Ticks were considered repelled if they fell from the finger without having crossed the upper boundary of the treated area or if they were on the untreated fingertip distal to the cloth. As in the in vitro bioassay, repellency was scored according to the locations of the ticks at 15 min. Before each bioassay JFC washed his index finger with soap and rinsed with water.

Experimental design

In vertical filter paper bioassays, amyris oil was tested against *I. scapularis* at 103, 51, 26, 13 and 0 µg oil/cm² paper ($n = 30$ ticks per concentration) and against *A. americanum* at 827, 413, 207, 103, 51, 26, and 0 µg oil/cm² paper to observe dose related responses ($n = 40$ ticks per concentration). Elemol was tested in vertical filter paper bioassays against *A. americanum* at 310, 155, 78, and 0 nmoles compound/cm² paper ($n = 30$ ticks per concentration). Amyris oil at 827 µg compound/cm² paper, was tested against *A. americanum* in vertical filter paper bioassays 2 and 4 h after application of the test solutions ascertain duration of activity.

In fingertip bioassays, amyris oil was tested against *I. scapularis* at 413, 207, 103, 51, 26, and 0 µg oil/cm² cloth and against *A. americanum* at 827, 413, 207, 103, and 0 µg oil/cm² cloth to observe dose related responses ($n = 30$ ticks per concentration). Elemol was tested in fingertip bioassays against *I. scapularis* at 155, 78, 39, 19, 10 and 0 nmoles compound/cm² cloth and against *A. americanum* at 775, 620, 310, 155, 78, 39, and 0 nmoles compound/cm² cloth ($n = 30$ ticks per concentration).

For comparative purposes deet was tested in fingertip bioassays with amyris oil against *I. scapularis* at 51 and 13 µg oil/cm² cloth and with elemol against *A. americanum* at 775 and 78 nmole compound/cm² cloth ($n = 30$ ticks per concentration).

Statistical analysis

The data collected were binomial in nature (ticks were either repelled or not, ticks either dropped or not) so fit in statistical framework of generalized linear models (McCullagh and Nelder 1989). We also found that there was often a day effect (“repellency” varied somewhat from day-to-day), which we included in the analyses as a random effect. In addition, for some analyses, there appeared other unknown sources of variation resulting in over-dispersed data (accommodated by using a quasi-binomial rather than binomial distribution, with an additional over-dispersion parameter). We used the R software (R Development Core Team 2009) with the lme4 package (Bates and Maechler 2009) to estimate models and test compound differences.

Since the software to calculate fiducial confidence limits (inverse regression) on dose has not been developed for generalized linear mixed models, we calculated these limits allowing for general overdispersion (but not specific random effects) using the dose.p function of the R MASS package (Venables and Ripley 2002) after fitting a generalized linear model in the quasi-binomial distribution family.

We found that by taking the square root of the dose the fit to the logit of the proportion was consistently more linear than any other transformations on dose, so we used square root transformed dose in all analyses.

Results

Amyris oil and elemol repelled both species of ticks in all bioassays. Figure 1 depicts the dose related responses of *A. americanum* and *I. scapularis* nymphs to elemol in fingertip and vertical filter paper bioassays and Fig. 2 shows the ticks’ responses to amyris oil. In wrapped-fingertip bioassays, all *I. scapularis* nymphs were repelled by 155 nmole elemol/cm² cloth and 97.3 and 100% of *I. scapularis* were repelled by 413 and 207 µg oil/cm² cloth of amyris oil, respectively. Higher concentrations of elemol and amyris oil were needed to repel *A. americanum* than *I. scapularis*. For example, 1,550 nmole elemol/cm² cloth were needed to repel 97.3% of *A. americanum* nymphs in fingertip bioassays. For direct comparison, two concentrations of deet were tested along with amyris oil in a series of wrapped-fingertip bioassays against *I. scapularis* and in a series of wrapped-fingertip bioassays with elemol against *A. americanum*. Deet and elemol did not differ significantly ($P = 0.558$) in their effectiveness against *A. americanum* in the fingertip bioassays. Against *I. scapularis*, deet was significantly ($P = 0.001$), but not greatly more repellent than amyris oil (Fig. 1). For both analyses, there was no significant interaction between concentration and compound.

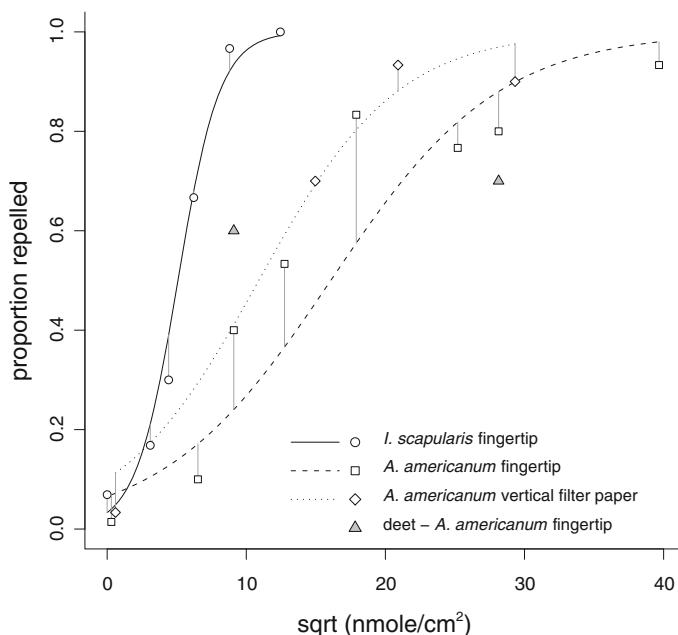


Fig. 1 Responses of *Ixodes scapularis* and *Amblyomma americanum* nymphs to elemol, deet and an ethanol control in vertical filter paper and wrapped-fingertip bioassays. Deet tested at two concentrations in fingertip tests for purposes of comparison

Table 1 shows the square roots of the EC₅₀ and EC₉₅ values for amyris oil in vertical filter paper and wrapped-fingertip bioassays for both species of ticks and for elemol in vertical filter paper bioassays against *A. americanum* and fingertip bioassays against both species. The data were out of the range of the model to calculate an EC₉₅ for amyris oil against *A. americanum*. Nymphs of *A. americanum* also exhibited greater variability in within dose responses compared to *I. scapularis*.

Amyris oil showed some prolonged repellent activity. In vertical filter paper bioassays, 827 µg amyris oil/cm² paper repelled 80% of *A. americanum* nymphs 2 h after application and 55% at 4 h after application.

The proclivity of *A. americanum* nymphs to drop off vertical surfaces treated with repellent is obvious in Fig. 3. In contrast, the nymphs of *I. scapularis* were also repelled by the elemol, but tended to remain on the untreated tip of the finger where they had been placed at the start of the test.

Discussion

Deet is often considered the standard to which other repellents are held for comparison. Carroll et al. (2004, 2007) and Zhang et al. (2009) tested deet in the same wrapped-fingertip and vertical filter paper bioassays using ticks from the same sources as in this study. For fingertip bioassays using nymphal *I. scapularis*, Carroll et al. (2007) reported an EC₅₀ and EC₉₅ of 23.9 and 58.4 n mole deet/cm² cloth, respectively, which compare to an EC₅₀ and EC₉₅ of 26.6 and 94.34 for elemol in this study (EC values presented as square roots in Table 1). Similarly, Zhang et al. (2009), who used the same wrapped-fingertip

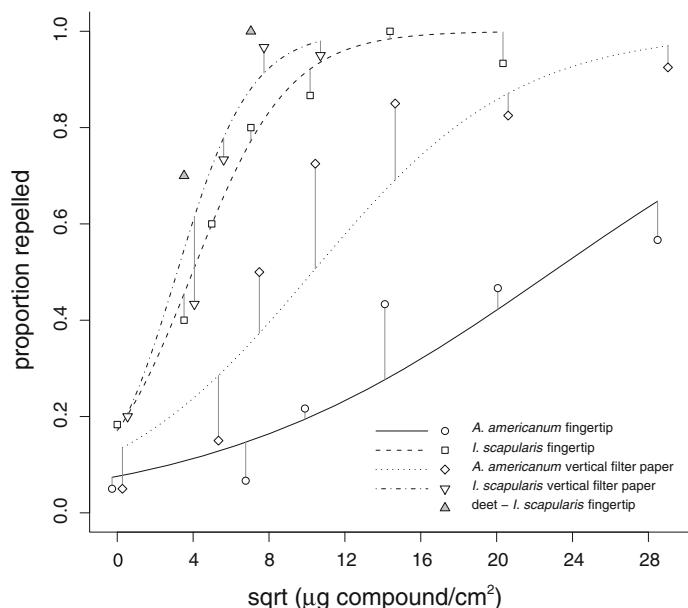


Fig. 2 Responses of *Ixodes scapularis* and *Amblyomma americanum* nymphs to amyris oil, deet and an ethanol control in vertical filter paper and wrapped-fingertip bioassays. Deet tested at two concentrations in fingertip tests for purposes of comparison

Table 1 Square roots of effective concentrations—of elemol and amyris oil against *Ixodes scapularis* and *Amblyomma americanum* nymphs in in vitro (vertical filter paper) and in vivo (cloth-wrapped fingertip) bioassays

Repellent	EC	Vertical filter paper bioassay		Wrapped fingertip bioassay	
		<i>I. scapularis</i>	<i>A. americanum</i>	<i>I. scapularis</i>	<i>A. americanum</i>
Mean μg/cm²					
Amyris oil	EC ₅₀	3.154 (0.418) ^a	9.043 (1.000)	4.199 (0.791)	22.912 (2.281)
	EC ₉₅	9.061 (0.927)	23.548 (2.654)	13.768 (2.036)	Outside range of data
Mean nmole/cm²					
Elemol	EC ₅₀		10.935 (1.809)	5.157 (0.399)	14.783 (1.232)
	EC ₉₅		26.107 (3.105)	9.713 (0.914)	33.494 (3.003)

^a Standard errors in parentheses

bioassay and ticks from the same source as this study, reported that 78 nmole deet/cm² cloth repelled all *I. scapularis* nymphs tested, but concentrations as high as 775 nmole deet/cm² cloth did not repel more than 80% of *A. americanum*. In this study, the estimated EC₅₀ and EC₉₅ for elemol against *A. americanum* were 218.5 and 1,121.8 nmole/cm² respectively, but in direct comparison, elemol did not differ in repellency from deet. Thus, it appears that elemol is as effective a repellent of *A. americanum* as is deet, but is somewhat less effective against *I. scapularis* than deet. Carroll et al. (2005) found that, in fingertip bioassays with cloth wrapped once around the finger, 1.6 μmole deet/cm² cloth repelled 95% of *I. scapularis* nymphs and 85% of *A. americanum* nymphs. In this study,

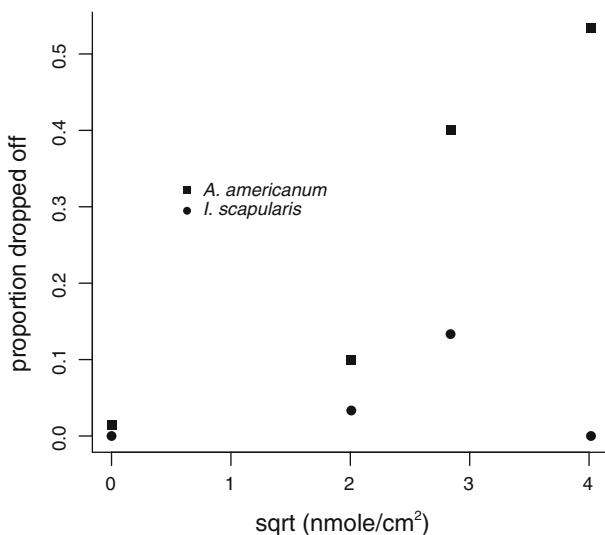


Fig. 3 Proportions of *Ixodes scapularis* and *Amblyomma americanum* nymphs that dropped from finger within 15 min, in response to three concentrations of elemol in doubly-wrapped fingertip bioassay. In contrast to *A. americanum*, nearly all *I. scapularis* were repelled at the two highest concentrations, but high proportions remained on the untreated fingertip

deet repelled significantly greater proportions of *I. scapularis* than amyris oil in wrapped fingertip bioassays, but as seen in Fig. 2, the difference was not great. Although we made no direct comparison of deet and amyris oil against *A. americanum* in this study, the reported EC₅₀ of 1.30 µmole deet/cm² paper for the vertical filter paper bioassay (Carroll et al. 2004) and 775 nmole deet/cm² cloth for 80% repellency in the wrapped fingertip bioassay (Zhang et al. 2009), suggest that when compared to deet amyris oil may be less effective than elemol.

Both amyris oil and elemol repelled *I. scapularis* nymphs at lower concentrations than were needed to repel *A. americanum* nymphs. This discrepancy between the responses of these species has been observed in previous studies in which fingertip and vertical filter paper bioassays were used to test deet, SS220, callicarpenal, intermedeol, and isolongifolenone (Carroll et al. 2004, 2005, 2007; Zhang et al. 2009). The responses of *A. americanum* to elemol and amyris oil also tended to be characterized by greater within dose variability than those of *I. scapularis*. Nymphs of *A. americanum*, unlike *I. scapularis*, often respond to repellents (e.g., deet, SS220) on vertical surfaces by dropping off them (Carroll unpublished data). In contrast, *I. scapularis* tend to withdraw from repellent-treated areas, and if the adjacent untreated surface is limited in size, as in the fingertip and vertical filter paper bioassays, the nymphs remain on the untreated surface. Drop off and back off behavior is well illustrated in the responses of *A. americanum* and *I. scapularis* to elemol in fingertip bioassays (Fig. 3). In terms of human protection, it is preferable that a tick drop off than for it to sequester itself on untreated or poorly treated skin or clothing.

Although the fingertip bioassay we used might be considered an *in vivo* test because a human subject was involved, it did not involve application of repellent to human skin. The treatment on the outer of two layers of cloth did not contact the skin. Absorption of the repellent by the skin and interactions with epidermal chemicals could result in different repellent activity. The double-wrapped finger bioassay does include the full array of host

stimuli and should elicit normal host acquisition and attachment site seeking behavior in the tick subjects. A useful feature of the double-wrapped finger bioassay is that it protects the human subject from dermal exposure to candidate repellents. The in vitro vertical filter paper test results are supportive of the fingertip bioassay findings, but in the three cases, in which both in vitro and in vivo bioassays were conducted using the same tick species and same repellent, higher concentrations of amyris oil or elemol were needed in the fingertip bioassay to achieve the same level of repellency as in the filter paper bioassay (Figs. 1, 2). An obvious difference between the bioassays is that ticks in the filter paper bioassay receive chemical (CO_2), physical and visual cues (Phillis and Cromroy 1977) from the observer, but tactile and likely other host-associated cues are lacking. Another important difference is that Whatman No. 4 filter paper absorbs more of the test solution than the organdy. More than twice the volume/cm² of test solution was applied to the filter paper, but it is unknown how much, if any, was absorbed too deeply into the paper to affect the ticks.

The concentrations at which elemol and amyris essential oil repelled *I. scapularis* and *A. americanum* in our bioassays and the concentrations which Paluch et al. (2009) found repelled *A. aegypti* mosquitoes are low enough to indicate that elemol and amyris oil should be investigated more thoroughly for their potential as marketable repellents.

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